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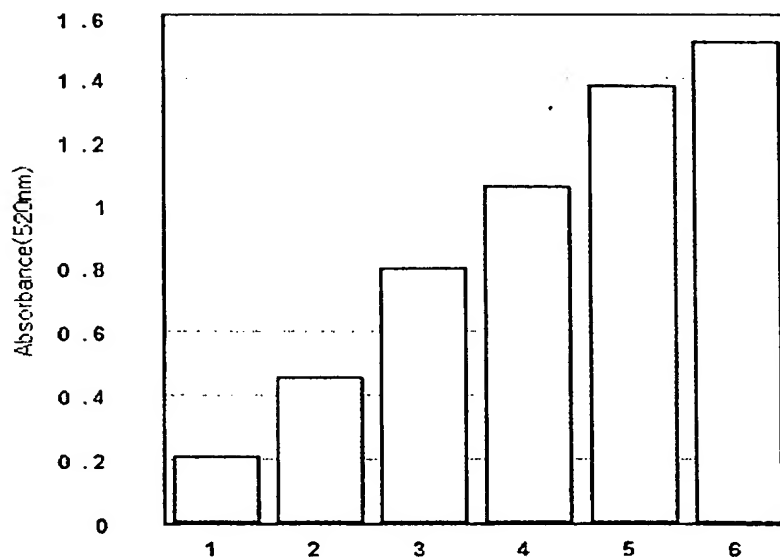
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(54) Title: COMPOSITION FOR DETECTING PEPTIDOGLYCAN, AND DIAGNOSTIC KIT DETECTING PEPTIDOGLYCAN



(57) Abstract: The present invention relates to a composition for selectively detecting an extremely small amount of peptidoglycan in sample, a preparation method of the composition, and a detection kit for peptidoglycan. It is possible to quantify a small amount of peptidoglycan contained in human blood, tissue, body fluid, water or food, and to diagnose an infection of microorganism with peptidoglycan as a component of cell wall using the composition and the detection kit. In addition, the composition can be applied for a diagnosis reagent of detecting an infection of Gram-positive bacteria in animal or human being in advance, and thus, can be used for the prevention or treatment of food poisonings and Bacterial sepsis.

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COMPOSITION FOR DETECTING PEPTIDOGLYCAN, AND
DIAGNOSTIC KIT DETECTING PEPTIDOGLYCAN

BACKGROUND OF THE INVENTION

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CROSS REFERENCE TO RELATED APPLICATION

This application claims priority to and the benefit of Korean Application Nos. 10-2001-31890 and 10-2002-31856, filed on June 8, 2001
10 and June 7, 2002 in the Korean Intellectual Property Office, the entire disclosure of which are incorporated herein by reference.

(a) Field of the Invention

The present invention relates to a composition for detecting
15 peptidoglycan, and a diagnostic kit for peptidoglycan using the same.

(b) Description of the Related Art

The infection of the pathogenic Gram-positive bacterium constitutes a large portion of widely-spread bacterial infection in hospitals. Food poisonings and bacterial sepsis caused by Gram-positive bacterium are
20 lethal diseases. A rapid detecting system for Gram-positive bacteria in clinical samples such as blood, tissue, and urine, and food is necessary. According to a conventional technique, it takes a few days to detect the bacteria. As foods contaminated with the bacteria will be distributed during this detection period, additional consumers may be infected.

25 It is possible to detect Gram-positive bacteria living in various types of samples in small amounts by detecting and quantifying the peptidoglycan. The peptideglycan is a kind of glycoprotein polymer constituting bacterial cell walls and contains N-acetylmuramic acid or N-glycosylmuramic acid and D-amino acid in outer cell wall of Gram-positive bacteria.

30 Accordingly, a detecting and measuring method of the peptidoglycan

can be applied for testing the safeness of the medicine, detecting microorganism in food and water, and performing diagnosis of infectious disease.

It is reported that a prophenoloxydase system of insect can detect
5 selectively a small amount of lipopolysaccharide (LPS), peptidoglycan, and
beta-1,3-glucan, in which zymogen-type prophenoloxydase is converted to
phenoloxidase in active form through cascade reactions to amplify the signal
more than 1,000 times. However, the prophenoloxydase system detects
all components including lipopolysaccharide, peptidoglycan, and beta-1,3-
10 glucan. Therefore, a system for selectively detecting any one of them is
necessary.

A prophenoloxidase, which exists in insect body with complete
metamorphosis, is activated to phenoloxidase through cascade reaction on
the beta-1,3-glucan or lipopolysacchride. The prophenoloxidase reaction
15 system consisting of a series of cascade reaction steps can be easily
activated to phenoloxidase system to produce melanin by using catechol
amines when it is exposed to exterior factors such as a pathogenic
microorganism and materials, or interior factors derived from degranulation
of interior hemocyte, etc. Thus, it is difficult to extract the prophenoloxidase
20 system from the insect body (Ashida and yoshida, (1988), Insect. Biochem.
18, 11-19).

Ashida et al. reported in Eur. J. Biochem, 188, 507-515 (1990) that
bivalent ion plays an important role in activating prophenoloxidase system by
introducing a composition which recognizes beta-1,3-glucan separated from
25 mosquito larva, and disclosing that prophenoloxidase system of insects
requires Ca^{2+} for its activation.

A composition and method for detecting peptidoglycan was disclosed
in U.S. Patent No. 4,970,152 where a protein reacting with beta-1,3-glucan
was removed from silkworm plasma to produce a reagent for specifically
30 detecting a peptidoglycan. However, the addition of Ca^{2+} is required for
activating a phenoloxidase system on peptidoglycan. In other word,

according to U.S. Patent No. 4,970,152, inhibition of the phenoloxidase system activation by adding Ca^{2+} is necessary when obtaining a phenoloxidase composition from the insect body fluid and triggering a color reaction on the peptidoglycan as a substrate with the composition.

5 Also, U.S. Patent No. 5,747,277 disclosed a SLP reagent. However, this reagent detected beta-1,3-glucan and peptidoglycan at the same time; therefore, it did not show a specific reaction to only peptidoglycan. As result, this reagent cannot be used for the detection of only peptidoglycan.

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SUMMARY OF THE INVENTION

Considering the shortcomings of the prior arts, the present invention provides a composition for selectively detecting a peptidoglycan.

This invention also provides a preparation method of the composition for detecting peptidoglycan.

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This invention further provides a method for selectively detecting peptidoglycan using the composition.

This present invention further still provides a detection kit for selectively detecting peptidoglycan using the composition.

BRIEF DESCRIPTION OF THE DRAWINGS

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The accompanying drawings, which are incorporated in and constitute a part of the specification, illustrate an embodiment of the invention, and, together with the description, serve to explain the principles of the invention.

Fig. 1 is a graph showing a phenoloxidase activity on beta-1,3-glucan or peptidoglycan with or without the addition of Ca^{2+} .

Fig.2 is a graph showing a phenoloxidase activity of plasma fraction B of *Galleria mellonella* larvae reacted with lipopolysaccharide, beta-1,3-glucan, or peptidoglycan.

Fig. 3 is a graph showing a phenoloxidase activity of plasma fraction B of *Galleria mellonella* larvae reacted with peptidoglycan in various

30

concentrations.

Fig. 4 is a graph showing phenoloxidase activities of plasma fraction B of *Galleria mellonella* larvae, hemocyte lysate, and mixture thereof reacted with beta-1,3-glucan, or peptidoglycan as a substrate.

5 Fig. 5 is a graph showing phenoloxidase activity of solution containing plasma fraction B with protein amount of 600 μ g, and hemocyte lysate with protein amount of 200 μ g of *Galleria mellonella* larvae which are reacted with peptidoglycan.

10 Fig. 6 is a graph showing a phenoloxidase activity of solution containing plasma fraction B and hemocyte lysate of *Galleria mellonella* larvae reacted with peptidoglycan in various concentrations.

Fig. 7 is a standard curve showing a phenoloxidase activity of solution containing plasma fraction B and hemocyte lysate of *Galleria mellonella* larvae reacted with 20ng/ml of peptidoglycan.

15 Fig. 8 is a graph showing a phenoloxidase activity of solution containing plasma fraction B and hemocyte lysate of *Galleria mellonella* larvae reacted with lipopolysaccharide, beta-1,3-glucan, or peptidoglycan as a substrate

20 DETAILED DESCRIPTION AND THE PREFERRED EMBODIMENTS

The present invention is directed to a composition for detecting peptidoglycan in a sample, a preparation method of the composition, and a detection kit for detecting peptidoglycan using the same.

25 In the present invention, a phenoloxidase system is intended to mean a system which is in *Galleria mellonella* larvae, and can be activated and converted to phenoloxidase by peptidoglycan.

In the present invention, a phenoloxidase composition is intended to mean a composition which comprises parts of phenoloxidase system or whole phenoloxidase system, and has a phenoloxidase activity on
30 peptidoglycan.

In the present invention, a phenoloxidase system of *Galleria*

mellonella does not require Ca^{2+} for its activation. Moreover, the addition of Ca^{2+} causes the suppression of the phenoloxidase activity.

The present invention provides a composition for detecting a peptidoglycan comprising extract of insect body fluid which has a phenoloxidase activity on peptidoglycan without the addition of Ca^{2+} . The extract of insect body fluid can be a plasma solution separated from insect body fluid. Compared with the composition containing only the plasma solution, the composition that further comprises hemocyte lysate in addition to the plasma solution allows detection of peptidoglycan that is small in number despite smaller amount of plasma solution is used. Thus, the composition comprising the plasma solution and hemocyte lysate is preferred.

The composition comprising plasma solution of insect body fluid and hemocyte lysate can be prepared by separating or without separating the hemocyte. In other word, the composition comprising plasma solution of insect body fluid and hemocyte lysate can be prepared by separating the hemocyte from the body fluid, lysing the hemocyte, and then adding the hemocyte lysate to the plasma solution. Or, the hemocyte lysate or partially-purified hemocyte lysate can be added to the partially purified plasma. Alternatively, when the hemocyte contained in the body fluid is not separated, the hemocyte can be broken down in part or as a whole to produce the solution. For examples, the sonification or high-speed centrifugation can be carried out on the body fluid or separated hemocyte. Also, the plasma solution, hemocyte lysate, and their mixture can be used in diluted form with addition of buffer solution, or in concentrated form by conventional concentration method. In the present invention, the hemocyte is intended to mean precipitation which can be obtained by removing the plasma solution from the insect body fluid. The hemocyte lysate can be a lysate prepared by breaking-down the hemocyte with addition of solvent, more preferably by obtaining supernatant form of the lysate.

In the composition for detecting peptidoglycan according to the present invention, the extract of insect body fluid is preferably derived from

Galleria mellonella larvae. That is, the composition comprises a part or whole of phenoloxidase system of *Galleria mellonella*, e.g. prophenoloxidase system. *Galleria mellonella* is short-lived with a life span of two months more or less that is considerably shorter than most of other insects', and has
5 strong multiplication capability allowing easy large-scale breeding means to yield a large amount of body fluid.

The plasma solution contained in insect body fluid can be obtained by treating *Galleria mellonella* larvae with solvent or buffer solution to produce factions, and by selecting the fraction showing a phenoloxidase
10 activity on peptidoglycan without addition of Ca^{2+} . Preferably, the solvent or buffer solution comprises sufficient amount of chelating agent to chelate Ca^{2+} in a sample or separation processes. More preferably, the fraction can be prepared by column chromatography. For example, the column can be filled with a sugar resin or vinyl resin as a carrier.

15 The composition for detecting peptidoglycan according to the present invention can be used for detecting the infection with Gram-positive bacteria, such as *Staphylococcus*, *Streptococcus*, *Pneumococcus*, and *Corynebacterium diphtheriae* in an individual.

In another aspect of the present invention, the preparation method of
20 composition comprising extract of insect body fluid which shows a phenoloxidase activity on peptidoglycan without the addition of Ca^{2+} . The preparation method of the present invention comprises obtaining plasma solution from the body fluid of *Galleria mellonella* larvae, treating the plasma with solvent or buffer solution to produce fractions, and selecting the fraction
25 showing a phenoloxidase activity on peptidoglycan.

In the preparation method, it is preferable to use an anticoagulant buffer solution for obtaining plasma from the body fluid of *Galleria mellonella* larvae. The anticoagulant can be any buffer solution capable of suppressing coagulation of body fluid, and especially citric acid buffer solution is preferred.
30 The anticoagulating buffer solution further comprises an inhibitor which can irreversibly inhibit a serine protease. The inhibitor of serine protease can be

any inhibitor which can irreversibly inhibit serine protease, thereby obtaining phenoloxidase fraction from the body fluid of *Galleria mellonella* larvae. Preferably, the inhibitor can include p-(aminoginophenyl)-methanesulfonylfluoride(p-APMSF), phenylmethanesulfonylfluoride(PMSF),
5 and diisopropylfluorophosphate(DFP). The concentration of inhibitor can be 0.2mM or more. When obtaining the insect body fluid, the chelating agent can be added to the anticoagulant for inhibiting coagulation of cells, and preventing activation of phenoloxidase system.

For example, a process of treating the plasma sample with solvent or
10 buffer solution in the preparation method can be carried out by a column chromatography.

There is no limitation on solvent or buffer solution used in the separation process. The anticoagulant can be used for obtaining the plasma solution from the insect. Preferably, by adding the chelating agent to the
15 anticoagulant, a desirable phenoloxidase composition can be obtained by inhibiting reactions associated with protein coagulation.

The chelating agent which is sufficient for chelating calcium ion contained in plasma sample and separation processes can include any kind of known chelating agents without limitation, for examples EDTA, EGTA,
20 citric acid, etc. The chelating agent can be used in various amounts, depending on the kind of subject insect, and separation conditions such as the kind of column, and solvent. A preferred amount of chelating agent can be a sufficient amount for chelating Ca^{2+} contained in separating processes. Accordingly, a person having an ordinary skill in the field can determine a
25 suitable amount of chelating agent without excess experimental efforts.

For example, the treating process of plasma of *Galleria mellonella* larvae with solvent or buffer solution can be carried out by a column chromatography in which the plasma can be loaded on column filled with resin, and eluted with solvent or buffer solution like an anticoagulating buffer
30 solution to produce fractions. Only column chromatography provides a composition for specifically detecting peptidoglycan without complicated

purification processes such as affinity chromatography.

The resin used in the column chromatography can be sugars such as monosaccharide or polysaccharide as a support, and preferably includes sugar resins such as agarose or dextran, and vinyl resin. For examples,
5 Sephadex or Toyoperal can be used.

The composition of the present invention can be used for specifically detecting peptidoglycan, and thus can be used for the diagnosis of bacterial infection containing peptidoglycan in cell walls.

Accordingly, the present invention provides a detection method for
10 the presence of peptidoglycan in a sample. The detection method comprises obtaining sample from subject matter, adding a composition showing a phenoloxidase activity on peptidoglycan in the absence of Ca^{2+} , and measuring the phenoloxidase activity of the sample. The composition showing a phenoloxidase activity is intended to include the composition for
15 detecting peptidoglycan. In an specific embodiment of the present invention, the plasma of *Galleria mellonella* larvae can be recovered in the presence of chelating agent with sufficient amount of chelating Ca^{2+} contained in plasma sample and separating processes, and then, the plasma can be treated with solvent which contains chelating agent in sufficient amount of chelating Ca^{2+}
20 contained in plasma sample and separating process to produce fractions. A fraction having a phenoloxidase activity on peptidoglycan can be selected by treating the fractions in an absence of Ca^{2+} to produce the composition of the present invention.

In the detection method of peptidoglycan according to the present
25 invention, the test subject can be those spread in the surroundings, such as animals including human being and live organisms. For example, the detection method includes a diagnosing method for Gram-positive bacteria by obtaining blood from the test subject and detecting peptidoglycan. In another example, it is possible to diagnose the infection of bacteria
30 containing peptidoglycan in cell walls, such as Gram-positive bacteria by obtaining water from the breeding field and detecting peptidoglycan.

As desired, to improve the specificity of diagnosis for bacterial infection, it is possible to remove lipopolysaccharide from the test sample. For example, it is possible to remove lipopolysaccharide by treating the test sample with agents capable of specifically binding to or precipitating the lipopolysaccharide such as polymyxin before diagnosing.

In the present invention, a conventional method or modified method, which is known as a measuring method of phenoloxidase activity, can be used in measuring the phenoloxidase activity. For example, by using coloring reaction with 4-methylcatechol /4-hydroxyprolineethylester (4-MC/4-HP), or melanin formation reaction with dopamine, the absorbance can be measured to provide phenoloxidase activity as explained below. The presence of peptidoglycan can be easily determined from the measurement of phenoloxidase activity.

In addition, the present invention provides a diagnosis kit for peptidoglycan. The diagnosis kit for peptidoglycan comprises the composition which has a phenoloxidase activity on peptidoglycan in the absence of Ca^{2+} . In the specific embodiment, the composition can be a composition which shows a phenoloxidase activity on peptidoglycan in the absence of Ca^{2+} . Preferably, the composition can be prepared by recovering plasma of *Galleria mellonella* larvae in the presence of chelating agent with sufficient amount of chelating Ca^{2+} contained in plasma sample and separating process, treating the plasma with solvent which contains chelating agent with sufficient amount of chelating Ca^{2+} contained in plasma sample and separating process to produce fractions, and selecting a fraction having the phenoloxidase activity on peptidoglycan by treating the fractions without the addition of Ca^{2+} .

The following examples are intended to further illustrate the present invention. However, these examples are shown only for better understanding of the present invention without limiting its scope.

Example 1: preparation of plasma and hemocyte from *Galleria*

mellonella larva

Larvae among *Galleria mellonella* larva that are about 2.5~3cm in length were selected and anesthetized on ice for 10-30 minutes. Then, anticoagulant buffer solution (pH 4.6) and 0.2mM of p-APMSF (Wako Co. Japan) was injected to the second node from the head with 5ml of syringe having 23G needle. The 4-5 drops of body fluid was obtained by halfway slicing the second node from tail, injecting buffer solution with syringe. The anticoagulant buffer solution contains 15mM of NaCl, 30mM of trisodium citrate, 26mM of citric acid, and 20mM of EDTA.

50ml of body fluid was centrifuged at 4°C, 371 xg, for 20 minutes to produce supernatant referred as "plasma", and precipitates referred as "hemocyte".

Example 2: Preparation of plasma solution

2-1: Pretreatment of extracted plasma solution

The plasma solution was treated with an ultra-filtration kit (membrane cut off. 3000) to obtain up to about 2 ml of concentrated plasma solution. In a condition where up to 5mM of calcium is added to the concentrated solution or no calcium is as to the concentration solution, the phenoloxidase activity of resulting concentrated solutions were measured, and showed in Fig. 1 as follows:

- 1: plasma solution,
- 2: plasma solution + 1 µg lipopolysaccharide (LPS),
- 3: plasma solution + 1 µg beta-1.3-glucan (BG),
- 4: plasma solution + 1 µg peptidoglycan(PG),
- 5: plasma solution + Ca²⁺,
- 6: plasma solution + 1 µg LPS + Ca²⁺,
- 7: plasma solution + 1 µg BG + Ca²⁺,
- 8: plasma solution + 1 µg PG + Ca²⁺.

The plasma solution was used to measure phenoloxidase activities

in 1 μg of LPS, peptidoglycan, and beta-1,3-glucan. From the result, the plasma solution of the present invention showed a low phenoloxidase activity on lipopolysaccharide (2nd rod), and a strong phenoloxidase activity on beta-1,3-glucan (3rd rod) and peptidoglycan (4th rod). Also, the system of *Galleria mellonella* larva can be activated in the absence of Ca^{2+} . In fact, it is found that this system is inhibited by the addition of Ca^{2+} .

2-2: Purification of a fraction specifically recognizing peptidoglycan

1.0 x 45 of column filled with Sephadex G-100 resin was equalized with anticoagulant buffer solution (pH 5.0). The concentrated sample (500mg of protein) was loaded on the equalized column, and then, was eluted with anticoagulant at a rate of 3.0 ml/test tube, and the eluted solution was taken to produce 3ml of 1 to 30 fractions.

Depending on the separation pattern of the proteins, the fractions were divided into Group A (fractions 1~6), Group B (fractions 7~10), and Group C (fractions 11~16). The groups B and C were concentrated with ultra-filtration Kit to be similar concentration of Group A. Before loading, the phenoloxidase activity of the sample, Groups A, B, and C on β -1,3-glucan, peptidoglycan, and lipopolysaccharide were measured and showed in Fig.2 as follows:

- 1: Plasma solution,
- 2: Plasma solution + 1ug of liposaccharide(LPS).
- 3: Plasma solution + 1 μg of beta-1,3-glucan(BG),
- 4: Plasma solution + 1 μg of peptidoglycan(PG),
- 5: Group A,
- 6: Group A + 1 μg LPS,
- 7: Group A + 1 μg BG,
- 8: Group A + 1 μg of PG,
- 9: Group B,
- 10: Group B + 1 μg of LPS,
- 11: Group B + 1 μg of BG,

- 12: Group B + 1 μg of PG,
13: Group C,
14: Group C + 1 μg of LPS,
15: Group C + 1 μg of BG,
5 16: Group C + 1 μg of PG.

Group B showed a phenoloxidase activity on only the peptidoglycan.

Example 3: Phenoloxidase activity of plasma fractions

The group B showed a phenoloxidase activity specifically for
10 peptidoglycan. This example was to test a sensitivity of Group B on
peptidoglycan.

Firstly, a peptidoglycan solution was made by suspending 1mg of
water-insoluble peptidoglycan (Fluka Co.) in 20mM TRIS solution (pH8.0).
100 μl of the suspension was added to 900 μl of TRIS solution, and then
15 was treated with sonification for a few seconds. 10 μl of the resultant
solution was used to measure the phenoloxidase activity.

To measure the phenoloxidase activity, modified Pye's
spectrophotometer method was used with 4-methylcatechol and 4-
hydroxyproline ethyl ester as a substrate. 1 μg /10 μl of peptidoglycan
20 suspension in TRIS buffer (pH 8.0) was mixed with 30 μl of sample (600 μg
of protein amount), and then, incubated at 30°C, for 5 minutes. As a negative
control, only 10 μl of 20mM TRIS buffer solution(pH8.0) was added. The
protein amount in the sample was determined by measuring an absorbance
at 280nm of wavelength, and meant the protein amount in total which were
25 contained in the sample prepared according the above method. Then, 442 μl
of 20mM TRIS buffer (pH8.0) was poured to test tube, added by 4-MC and 4-
HP to be 1mM, and 2mM, respectively, and adjusted to 500 μl of volume.
The resultant was incubated at 30°C for 20 minutes. Then, 100 μl of solution
was taken from test tube, diluted with 100 μl of anticolagulant (pH4.6), and

then true phenoloxidase activity was determined by measuring an absorbance of 2 times-diluted phenoloxidase activity at 520nm of wavelength with spectrophotometer, and multiplying the absorbance with dilution times.

When measuring the phenoloxidase activity under the condition of
5 calcium addition, 20 mM of TRIS buffer solution (pH 8.0) added by 5.65mM of CaCl_2 was used for determining the phenoloxidase activity according to the substantially same method as explained above.

30 μl of Group B solution prepared according to Example 2 (600 μg of protein amount) was used to determine the phenoloxidase activity
10 depending on the various concentration of water-soluble peptidoglycan without the addition of Ca^{2+} or with the addition of Ca^{2+} . The result was shown in Fig. 3 as follows:

- 1: Group B,
- 2: Group B + 3ng of peptidoglycan (PG),
- 15 3: Group B + 5ng of PG,
- 4: Group B + 10ng of PG,
- 5: Group B + 20ng of PG,
- 6: Group B + 30ng of PG.

It was confirmed that Group B solution could quantify the
20 peptidoglycan up to 3ng/ml of concentration.

Example 4: Hemocyte lysate of *Galleria mellonella* larvae

The hemocytes separated from body fluid as disclosed in Example 1 were added by 50mM of TIRS buffer (pH 6.5) including 1mM EDTA as much
25 as half of the volume of hemocyte treated with sonification for 2 minutes, and then, centrifuged at 4°C, 3,586 xg for 20 minutes to produce the supernatant, as called "Primary sample." The precipitate removed from the supernatant was added by TIRS buffer as much as half of the volume of hemocyte, and centrifuged one more time to produce the supernatant, referred as "Second
30 sample." The primary and second samples referred as "hemocyte lysate" were kept in a refrigerator at -80°C for the following use.

Example 5: Phenoloxidase activity and specificity of hemocyte lysate on peptidoglycan

To determine whether the hemocyte lysate can react with
5 peptidoglycan specifically, the phenoloxidase activity of hemocyte lysate was determined for its activity on beta-1,3-glucan and peptidoglycan, which is shown in Fig. 4. The following solutions were used for this example:

- 1: Plasma fraction B solution of Example 2 (Protein amount: 200 μg),
- 2: plasma fraction B solution (200 μg) + beta-1,3-glucan (1 μg),
- 10 3: plasma fraction B solution (200 μg) + peptidoglycan (1 μg),
- 4: hemocyte lysate (protein amount: 100 μg),
- 5: hemocyte lysate (100 μg) + beta-1,3-glucan (1 μg),
- 6: hemocyte lysate (100 μg) + peptidoglycan (1 μg),
- 7: plasma fraction B solution (200 μg) + hemocyte lysate (100 μg),
- 15 8: plasma fraction B solution (200 μg) + hemocyte lysate (100 μg) +
beta-1,3-glucan (1 μg)
- 9: plasma fraction B solution (200 μg) + hemocyte lysate (100 μg) +
peptidoglycan (1 μg)

As a result, when the plasma fraction B solution of Example 2
20 including anticoagulant (protein amount: 200 μg) was added by 1 μg of beta-1,3-glucan and 1 μg of peptidoglycan, respectively (2nd, and 3rd rods of Fig.4), the phenoloxidase activity was not detected. Also, when the hemocyte lysate solution (protein amount: 100 μg) was added by 1 μg of beta-1,3-glucan and 1 μg of peptidoglycan, respectively (5th, and 6th rods of Fig.4), the
25 phenoloxidase activity was not detected. However, when plasma fraction B solution (protein amount: 200 μg) was added by 100 μg of hemocyte lysate and 1 μg of peptidoglycan (9th rod), the increase in the phenoloxidase activity was detected. However, when plasma fraction B solution (protein amount: 200 μg) was added by 100 μg of hemocyte lysate and 1 μg of beta-1,3-

glucan (10th rod), the phenoloxidase activity was not detected.

Accordingly, the mixture of plasma fraction B solution and hemocyte lysate specifically recognized peptidoglycan and showed phenoloxidase activity. In addition, compared with only the plasma fraction B solution, the mixture showed the phenoloxidase activity despite of using a smaller amount of plasma fraction B solution. Thus, it is possible to detect peptidoglycan with a smaller amount of plasma fraction by adding the hemocyte lysate to the plasma fraction.

10 Example 6: Effect of hemocyte lysate on phenoloxidase activity

After reaction with 30 μ l of each solution of Plasma fraction B solution of Example 2 (protein amount: 600 μ g), plasma fraction B solution (protein amount: 600 μ g) + peptidoglycan (1 μ g), plasma fraction B solution (protein amount: 600 μ g) + hemocyte lysate (protein amount: 100 μ g), plasma fraction B solution (protein amount: 600 μ g) + hemocyte lysate (protein amount: 100 μ g) + peptidoglycan (1 μ g) for 15 minutes, the phenoloxidase activities of the solutions were determined according to Example 3.

The phenoloxidase activity was measured on the solution which was substantially the same as the above solutions except that the plasma fraction B solution of Example 2 (protein amount: 200 μ g) was added by anticoagulant. The result was shown in Fig. 5:

- 1: Plasma fraction B solution (600 μ g),
- 2: Plasma fraction B solution (600 μ g) + peptidoglycan (1 μ g),
- 3: Plasma fraction B solution (600 μ g) + hemocyte lysate (100 μ g),
- 25 4: Plasma fraction B solution (600 μ g) + hemocyte lysate (100 μ g) + peptidoglycan (1 μ g),
- 5: Plasma fraction B solution (200 μ g),
- 6: Plasma fraction B solution (200 μ g) + peptidoglycan (1 μ g),
- 7: Plasma fraction B solution (200 μ g) + hemocyte lysate (100 μ g),

8: Plasma fraction B solution (200 μg) + hemocyte lysate (100 μg) + peptidoglycan (1 μg).

As a result, while the fraction B solution with protein amount of 600 μg showed strong phenoloxidase activity on peptidoglycan (2rd rod), the
5 fraction B solution with protein amount of 200 μg showed low phenoloxidase activity on peptidoglycan (6th rod). Accordingly, the addition of the hemocyte lysate (protein amount: 100 μg) to plasma fraction B solutions increased the phenoloxidase activity. Both the solutions with protein amount of 600 μg and 200 μg had higher phenoloxidase activity (4th and 8th rods). Accordingly, it
10 was found that a component contained in hemocyte lysate activated the phenoloxidase specifically.

Example 7: Phenoloxidase activity of a composition comprising plasma fraction B solution and hemocyte lysate

15 30 μl of the solution (protein amount of fraction B: 200 μg , protein amount of hemocyte lysate: 100 μg) containing the plasma fraction B solution added by anticoagulant buffer solution (200 μg of protein amount of plasma fraction B) of Example 2, and the hemocyte lysate prepared in Example 4 was tested for the phenoloxidase activity on the various concentrations of
20 peptidoglycan. In order to obtain the standard curve, the phenoloxidase activity was determined according to the substantially same method as explained above except that the concentration of peptidoglycan was 2, 5, 10, or 20ng/ml.

2ng/ml, 20ng/ml and 200ng/ml of the peptidoglycan solutions were
25 prepared according to the substantially same method of Example 3, treated with 4-MC/4-HP coloring reaction at 30 °C for 1 hour, and then absorbance at 520nm was measured. The result of the experiment was shown in Fig. 6, and the standard curve was shown in Fig. 7. As shown in Fig. 7, the correlation constant between the peptidoglycan concentration and the
30 phenoloxidase activity was 0.98, thereby allowing detection of even small

amount of peptidoglycan.

Example 8: Specificity on peptidoglycan

To determine whether the plasma fraction B solution of Example 2 (protein amount: 200 μg) and hemocyte lysate (protein amount: 100 μg) have a specificity on peptidoglycan, the phenoloxidase activities on lipopolysaccharide and beta-1,3-glucan were measured on 30 μl of the plasma fraction B solution of Example 2 (protein amount: 200 μg) and hemocyte lysate (protein amount: 100 μg), respectively. 2ng/ml, 20ng/ml, and 200ng/ml of the substrate solutions containing beta-1,3-glucan were prepared by suspending beta-1,3-glucan in 20mM TRIS buffer solution (pH 7.6). 2ng/ml, 20ng/ml, and 200ng/ml of the substrate solutions containing lipopolysaccharide (LPS) were prepared by suspending lipopolysaccharide (Sigma Co.) in 20mM TRIS buffer solution (pH 7.6), and then sonicating for 2-3 minutes. The phenoloxidase activities were determined using the beta-1,3-glucan solution and the LPS solution as a substrate according to the method of Example 3. This result was then compared with the experiment where 20 ng/ml of peptidoglycan and was shown in Fig. 8.

As a result, 200 ng/ml of LPS and beta-1,3-glucan solution did not show any phenoloxidase activity despite the reaction time is increased. Thus, it was found that the phenoloxidase composition of the present invention could be used for specific detection of peptidoglycan.

The present invention makes it possible to quantify even a small amount of peptidoglycan contained in human blood, tissue, body fluid, water or food, and to diagnose infection of microorganism having peptidoglycan as a component of cell wall. In addition, the composition can be used as a diagnosis reagent for detecting an infection of Gram-positive bacteria in animal or human being in advance. Therefore, the composition can be used for prevention or treatment of food poisoning and Bacterial sepsis.

WHAT IS CLAIMED IS:

1. A composition for detecting a peptidoglycan comprising extract of insect body fluid which has a phenoloxidase activity on the peptidoglycan without the addition of calcium.
- 5 2. The composition according to claim 1, wherein the extract of insect body fluid is a plasma solution separated from insect body fluid.
3. The composition according to claim 1, wherein the extract of insect
10 body fluid comprises a plasma solution and hemocyte lysate of insect body fluid.
4. The composition according to claim 1, wherein the extract of insect body fluid is derived from *Galleria mellonella* larvae.
- 15 5. The composition according to claim 2, wherein the extract of insect body fluid is a fraction having a phenoloxidase activity on peptidoglycan without the addition of calcium selected from fractions which are prepared by treating plasma of insect body fluid with solvent or buffer solution.
- 20 6. The composition according to claim 3, wherein the extract of insect body fluid is a fraction having a phenoloxidase activity on peptidoglycan without the addition of calcium selected from fractions which are prepared by lysing hemocyte contained in insect body fluid containing plasma and
25 hemocyte, and treating with solvent or buffer solution.
7. The composition according to claim 3, wherein the extract of insect body fluid is a fraction having a phenoloxidase activity on peptidoglycan without the addition of calcium selected from fractions which are prepared by
30 adding hemocyte lysate or partially-purified hemocyte lysate to fractions obtained by treating plasma of *Galleria mellonella* larvae with solvent or

buffer solution.

8. The composition according to any one of claims 5, 6, and 7, wherein the solvent or buffer solution comprises a chelating agent in a sufficient
5 amount for chelating calcium ion existing in a sample and separation process.

9. The composition according to any one of claims 5, 6, and 7, wherein the fraction is a fraction prepared through column chromatography.

10 10. The composition according to claim 9, wherein the column is filed with a sugar resin or a vinyl resin.

11. A detection method of peptidoglycan comprising obtaining of sample from the test subject, adding the composition for detecting peptidoglycan
15 according to any one of claims 1 to 4 to the sample, and measuring a phenoloxidase activity.

12. The detection method according to claim 11, wherein the extract of insect body fluid is a fraction having a phenoloxidase activity on
20 peptidoglycan without the addition of calcium selected from fractions which are prepared by treating plasma of insect body fluid with solvent or buffer solution.

13. The detection method according to claim 11, wherein the extract of
25 insect body fluid is a fraction having a phenoloxidase activity on peptidoglycan without the addition of calcium selected from fractions which are prepared by lysing hemocyte in insect body fluid containing plasma and hemocyte, and treating with solvent or buffer solution.

30 14. The detection method according to claim 11, wherein the extract of insect body fluid is a fraction having a phenoloxidase activity on

peptidoglycan without the addition of calcium selected from fractions which are prepared by adding hemocyte lysate or partially-purified hemocyte lysate to fractions obtained by treating plasma of *Galleria mellonella* larvae with solvent or buffer solution.

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15. A detection kit for peptidoglycan comprising the composition for detecting a peptidoglycan of any one of claims 1 to 4.

16. The detection kit according to claim 15, wherein the extract of insect
10 body fluid is a fraction having a phenoloxidase activity on peptidoglycan without the addition of calcium selected from fractions which are prepared by treating plasma of insect body fluid with solvent or buffer solution.

17. The detection kit according to claim 15, wherein the extract of insect
15 body fluid is a fraction having a phenoloxidase activity on peptidoglycan without the addition of calcium selected from fractions which are prepared by lysing hemocyte in insect body fluid containing plasma and hemocyte, and treating with solvent or buffer solution.

20 18. The detection kit according to claim 15, wherein the extract of insect body fluid is a fraction a having phenoloxidase activity on peptidoglycan without the addition of calcium selected from fractions which are obtained by adding hemocyte lysate or partially-purified hemocyte lysate to fractions obtained by treating plasma of *Galleria mellonella* larvae with solvent or
25 buffer solution.

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR02/01086

A. CLASSIFICATION OF SUBJECT MATTER**IPC7 C12Q 1/26**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7 C12Q 1/26

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CA, PubMed, Delphion, "peptidoglycan", "phenoloxidase"

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|------------------------------------------------------------------------------------|-----------------------|
| X; Y | Yoshida, H. et al., J. Biol. Chem., 271(23), 13854-60, 1996. | 1,-3,5,6,8-18; 4,7 |
| Y | US 4,970,152 A (Wako Pure Chem. Ind., Ltd.), Nov. 13, 1990. | 1-18 |
| Y | JP 11196895 A2 (Seikagaku Kogyo Co., Ltd.), Jul. 27, 1999. | 1-18 |
| Y | JP 11178599 A2 (Wako Pure Chem. Ind., Ltd.), Jul. 06, 1999. | 1-18 |

☐ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance
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"&" document member of the same patent family

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR02/01086

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|-------------------------------------------|---------------------|----------------------------------------------------|-------------------------------------------------|
| US 4,970,152 A | Nov. 13, 1990 | EP 270039 A2 JP 63-141598 A2 JP 63-141599 A2 | Jun. 08, 1988 Jun. 14, 1988 Jun. 14, 1988 |
| JP 11196895 A2 | Jul. 27, 1999 | none | |
| JP 11178599 A2 | Jul. 06, 1999 | none | |